

What is the destiny of a threatened fish, *Ptychobarbus chungtienensis*, now that non-native weatherfishes have been introduced into Bita Lake, Shangri-La?

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ABSTRACT

Biological invasion is a pervasive negative force of global change, especially in its effects on sensitive freshwater ecosystems. Even protected areas are usually not immune. *Ptychobarbus chungtienensis* is a threatened freshwater fish now almost confined to Bita Lake, in the Shangri-La region of Yunnan province, China. Its existence is threatened by the introduction of non-native weatherfishes (*Misgurnus anguillicaudatus* and *Paramisgurnus dabryanus*) by an unusual method known as 'prayer animal release'. Periodic surveys revealed the ratio of invasive weatherfishes to *P. chungtienensis* has been increasing since the former species was first recorded from the lake in August, 2009. *Ptychobarbus chungtienensis* shows low genetic diversity in the relict Lake Bita population. Weatherfishes, however, have highly successful survival strategies. The degree of dietary overlap between the species is alarming and perhaps critical if food is found to be a limiting factor.

Keywords: Biological invasion; Threatened fish; Prayer animal release; Genetic diversity; Dietary

INTRODUCTION

Biological invasions have become a pervasive agent of global change not only in homogenizing the earth's biota (Lodge, 1993; Simberloff et al., 2013), but also in challenging the conservation of biodiversity and natural resources (TEEB, 2010). Freshwater ecosystems are particularly sensitive to such invasions (Villéger et al., 2011), where invasive species become one of the major

threats to the global freshwater biodiversity (Geist, 2011; Magurran, 2009). Although without enough attention, China is one of the countries suffering from serious biological invasions (Xu et al., 2006; Xing et al., 2016). When taking the intentional transplantation (Kang et al., 2013), escape of domestication stocks (Yang et al., 2011), and accompanied by other forms of introduction (Chen, 2010) into consideration, the biological invasion in the freshwater ecosystem in China is quite severe. Generally speaking, lacustrine ecosystem (lakes, ponds, etc.) are more sensitive than riverine ecosystem (rivers, streams, etc.), and the extinction, replacement of indigenous fish by introduced species is extremely common all over the lakes of China whether from plain lakes in the east (Chen et al., 2010), or plateau lakes in the west (Chen et al., 1998).

Bitahai Lake is a plateau freshwater lake in the Yunnan Bitahai Nature Reserve, which is a major part of the Putatso National Park. It is also the core area of the Bitahai wetland, one of the Wetlands of International Importance (Ramsar sites in 2004), with an elevation of about 3 568 m a.s.l. and a catchment area 159 hm² (Yang & Ji, 2010) (Figure 1A-C). The fish fauna in Bitahai Lake is extremely simple, *Ptychobarbus chungtienensis*, one of

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the plateau schizothoracinae fishes (Cyprinidae), was recorded as the only fish species (Figure 1D), which was considered to be extraordinarily unusual in the evolution of species and the formation of lakes (Chen & Chen, 2002). *Ptychobarbus chungtienensis* is a threatened freshwater fish that has been listed as Endangered on the China Species Red List (Pan et al., 2010; Wang & Xie, 2004). It was originally recorded on the Zhongdian Plateau of the Jinsha River drainage (upper Yangtze)

in China including Bita Lake, Napa Lake, Shudu Lake, the Naya, Xiaozhongdian and Geza Rivers¹ (Chen, 2010; Huang & Chen, 1986). However, according to field surveys in recent years, it has been almost extirpated from its original localities, and only Bita Lake contains a relative large population (Jiang et al., 2013). The fact that the *P. chungtienensis* is endemic to Bita Lake and is the only native species present makes this lake ecosystem unique.

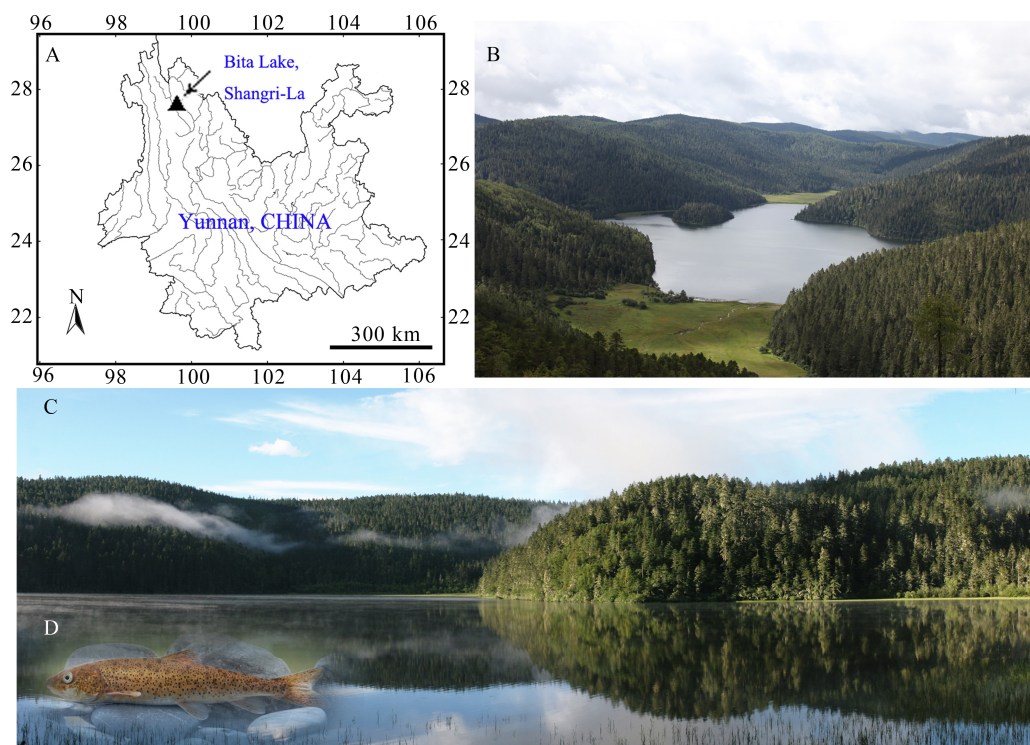


Figure 1 Bita Lake and the only native fish *P. chungtienensis* in it

A: the location of Bita Lake; B: the aerial view of Bita Lake from the Northern hillside; C: the nearby view of Bita Lake from the Northern lakeshore; D: the living photo of *P. chungtienensis*.

However, this special relationship of *P. chungtienensis* and Bita Lake has been interrupted by the introduction of non-native oriental weatherfishes species² at some point after 2009. The exotic species are generally threatening the native ones, particularly to those that have reduced populations with low genetic diversity (Magurran, 2009), and those in simple

ecosystems with vulnerable trophic cascades (Estes et al., 2011). The situation in Bita Lake is possibly one example of this. From 2008 to 2013, we carried out periodic surveys of the fishes in Bita Lake. This paper aims to provide a dynamic assessment of *P. chungtienensis* and the introduced weatherfishes by successive monitoring data, and investigate the potential risks to this threatened species through its intrinsic genetic diversity and the extrinsic competition for food with weatherfishes.

MATERIALS AND METHODS

Acoustic surveys using scientific Echo sounders have increasingly become valuable and frequently-used methods in fish assemblages monitoring and fishery management (Fernandes, 2009). A portable Echo sounder HONDEX HE-51C operating at a frequency of 200 kHz was used during the surveys. Echo signals were recorded in the afternoon of each

¹ The population in Geza River was considered as a subspecies named *P. chungtienensis gezaensis* before with distinct morphological variations (Huang & Chen, 1986), but we now tentatively treated it as a distinct sister species *P. gezaensis* according to the regular practice in fish taxonomy that no more use of the category of subspecies.

² These introduced oriental weatherfishes actually include two species with close relationships, similar morphology and ecological niches, *Misgurnus anguillicaudatus* and *Paramisgurnus dabryanus*. The two species were usually mixed in our samples so we simply refer to them as weatherfishes in this paper.

investigating day along the same route. Four non-overlapping transects (BT1, 2, 3, 4) were chosen to cover the different depths, crossing the relative shallow areas in the east (<3 m), the deep areas in the mid-south (>7 m), and most areas with an average depth of 4 m. Echo recordings were cumulated at each transect, and the averages of each transect at different times each year were calculated to produce the curve of annual fluctuations. The recordings of the four transects from different times were statistically compared using the one-way ANOVA test (SPSS package). Fish samples were captured at night at the same day in three sites of the lake body using traps, and three surrounding inlet creeks using a backpack electro-shocker. The numbers of *P. chungtienensis* and weatherfishes in each sampling were recorded to see the changes of fish composition. Most of the fishes were released the next morning, and only a few samples were fixed by formalin to do the length-weight relationship, age determination, sex ratio and gonad analyses (Jiang et al., 2013), and conduct genetic diversity and dietary composition analyses in this study.

The genetic diversity of the mitochondrial cytochrome *b* (Cyt *b*) gene sequence of *P. chungtienensis* was analyzed. Total DNA of 81 specimens from Bita Lake were purified from alcohol-preserved fin by the standard methods (Sambrook et al., 1989). The following cycling conditions were used in polymerase chain reaction (PCR): an initial denaturing step at 95 °C for 4 min; 38 cycles of denaturing at 94 °C for 1 min, annealing at 52 °C for 1 min, and an extension at 72 °C for 1 min; with a final extension step of 72 °C for 10 min. Amplification primers of Cyt *b* were adopted from other Cyprinidae species as L14724 (5'-GACCTTGAAAAACACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Xiao et al., 2001). All sequences have been deposited in GenBank (Accession No: KJ841795-KJ841875). Three sequences from GenBank (Accession No: FJ601043, AY463507, and AY463508, the last one from Xiaozhongdian River and the others from Bita Lake) were also downloaded and included in all of the analyses. One sequence of *P. gezaensis* (historically considered a subspecies) from the Geza River (GenBank Accession No. AY463506) was used as the outgroup to calculate the genetic distance and haplotype variations. Sequences were aligned with ClustalW (Thompson et al., 1994) and then calculated the pairwise distance (*p*-distance) in MEGA 6 (Tamura et al., 2013). The number of distinct haplotypes, haplotype diversity (*h*) (Nei, 1987) and nucleotide diversity (π) (Nei & Tajima, 1981) were calculated using DnaSP version 5.0 (Librado & Rozas, 2009) to estimate the standard indices of genetic diversity. A Median-Joining (MJ) approach (Bandelt et al., 1999) was performed to visualize relationships among haplotypes in Network v.4.6.1.2 (<http://www.fluxus-engineering.com/>).

Dietary composition of *P. chungtienensis* and the weatherfishes was analyzed to compare their food niche relationships and degree of dietary overlap. Stomachs of all fishes collected were excised and fixed in a 10% formalin solution to avoid further digestion, and the total length (TL) and body weight (BW) were recorded. Prey items were identified to the lowest possible taxonomic level and enumerated based on their main physical characteristics under a microscope. All

individuals and segments of each prey category were sorted dry and weighed jointly to the nearest 0.001 g. The percentage by number (%N), percentage by weight (%W), percentage frequency of occurrence (%F.O) and index of relative importance (IRI) were recorded to quantify the diet composition, and the overlap formula of Pianka (1973) and a modified *PS* index of Shorygin (1952) were used to assess the diet overlaps. These equations used to quantify the diet composition and assess the dietary overlap were listed below.

Percentage by number (N%)=(number (*n*) of individuals of a prey category/total number (*n*) of individuals among all prey categories)×100 (1)

Percentage by weight (W%)=(weight (*g*) of individuals of a prey category/total weight (*g*) of individuals among all prey categories)×100 (2)

Percentage frequency of occurrence (F.O%)=(number (*n*) of stomachs containing a prey taxon/total number (*n*) of stomachs containing prey)×100 (Bowen, 1996; Hyslop, 1980) (3)

IRI =%F.O (%N + %W) (Pinkas et al., 1971) (4)

where IRI is the index of relative importance, %N, %W and %F.O are these defined at (1, 2, 3).

$$O_{jk} = \sum P_{ij}P_{ik} / \sqrt{\sum P_{ij}^2 P_{ik}^2} \quad (\text{Pianka, 1973}) \quad (5)$$

where O_{jk} is the overlap index of Pianka which indicated the niche overlap of species *j* and *k*, P_{ij} and P_{ik} are the numerical proportions of the food item *i* used by the species *j* and *k* respectively.

$$PS = \sum_{\min} (a, b) \quad (\text{Ivlev, 1961}) \quad (6)$$

where *a* and *b* are the percentages by number of the prey common to the two predators, and the percentage similarity (*PS*) is a summation of the smaller of the values of *a* and *b* for each prey. The index ranges from 0 (no overlap) to 100 (complete overlap).

RESULTS

Population dynamics of *P. chungtienensis* and weatherfishes

Eight effective Echo sounder surveys were made between 2009 and 2012. The fluctuations of mean numbers each year in each transects can be seen in Figure 2A. Generally, the first two transects (BT1 & 2) recorded more individuals than that in the last two transects (BT3 & 4). It might be a result of the different characters of specific length and depth of each transect. These recordings of transects were not significantly different among different years (from 2009 to 2012, *P*=0.270), which shows no significant change in the quantity of fish in these years. A total of 12 samples were used to compare the population dynamics of *P. chungtienensis* and weatherfishes can be seen in Figure 2B. The catch ratios of weatherfishes to *P. chungtienensis* varied from 0 (no weatherfishes) before August 2009 to a maximum of 16.2 in August 2012.

Genetic diversity of *P. chungtienensis*

A 1 140 base pair fragment of Cyt *b* sequence was amplified, with six variable sites and three parsimony-informative sites.

The overall p-distance within *P. chungtienensis* was 0.001, and the mean distance between *P. chungtienensis* and *P. gezaensis* was 0.017. Seven unique haplotypes were identified in all 84 sequences. The h and π values inferred from individuals of *P. chungtienensis* were 0.666 and 0.000 81, respectively. The Median-Joining network revealed a simple haplotype relationship for *P. chungtienensis* (Figure 3). There were at least 20 mutations isolating *P. chungtienensis* and its sister species *P. gezaensis*. Haplotype 1 (H1) was a relatively ancestral haplotype of *P. chungtienensis*, because it was shared by individuals from Bita Lake and the Xiaozhongdian River. Another comparable haplotype, H2, was shared by 37 individuals, and all other haplotypes radiated from H2 by only one or two mutations.

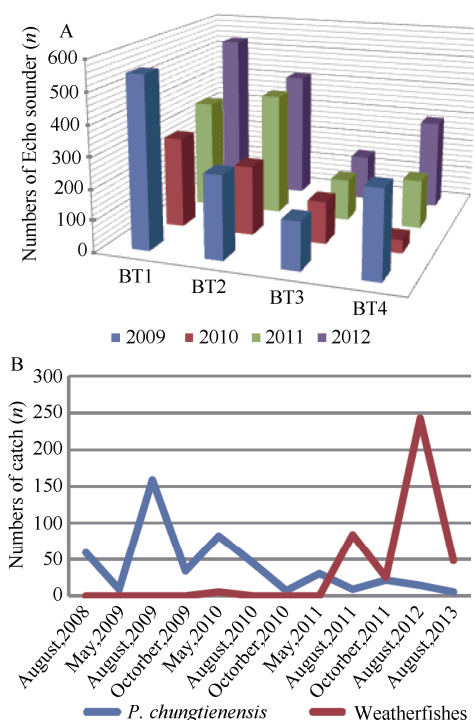


Figure 2 Field surveys of *P. chungtienensis* and weatherfishes in Bita Lake

A: annual fluctuations in fish numbers (n) from Echo sounder recordings in four transects from 2009 to 2012; B: catch numbers (n) of *P. chungtienensis* and weatherfishes from 2008 to 2013.

Diet composition and overlapping of *P. chungtienensis* and weatherfishes

A total of 105 individuals of *P. chungtienensis* (90–221 mm in TL and 11.9–134 g in BW) were used to investigate stomach contents, of which 92 (87.6%) individuals contained food. The sample of weatherfishes included 125 individuals (76–203 mm in TL and 3.8 to 74.5 g in BW) which 119 (95.2%) had food. The diet composition (listed by N%, W%, F.O% and IRI) of *P. chungtienensis* and weatherfishes in Bita Lake is given in Table 1. According to the IRI values for *P. chungtienensis*, algae (*Spirogyra* and *Zygnema*) were most important, followed in

order by Trichoptera (*Philopotamus*), Amphipoda (*Gammarus bitaensis*), and aquatic plants. These four items together represented 97.6% of the food intake. For weatherfishes, the sequence was Amphipoda (*Gammarus bitaensis*), algae (*Spirogyra* and *Zygnema*), and Diptera (larvae of *Chironomus*). These three items comprised 95.7% of the relative dietary importance. The overlap index of Pianka between *P. chungtienensis* and weatherfishes was 0.69. According to a study of trophic relationships of fishes in an Ontario temperate lake (Keast, 1978), the O_{jk} value of 0.3 or less was insignificant, and one of 0.7 or more was considered high overlap of prey composition. The PS index of Shorygin between *P. chungtienensis* and weatherfishes was 79.16%, which is much greater than the critical point of 60% (obvious similarity of prey composition (Blaber & Bulman, 1987)). Both the overlap index of Pianka and a modified PS index of Shorygin suggested a high degree of dietary overlap between *P. chungtienensis* and weatherfishes.

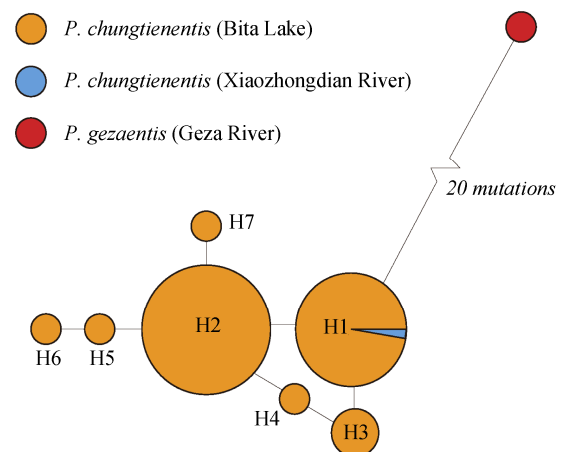


Figure 3 Median-joining network of *P. chungtienensis* based on seven haplotypes (H1 to H7) of Cyt *b* gene from 84 individuals in Bita Lake and the Xiaozhongdian River, with comparing to its sister species *P. gezaensis* in the Geza River

DISCUSSION

Non-native species have been introduced into new ecosystems primarily through human activity, either deliberately or unintentionally (Gozlan et al., 2010). The approach of deliberately introductions is mainly from societal demands for fish products for food aquaculture (51%), ornamental fish (21%), sport fishing (12%) and fisheries (7%) (Gozlan, 2008). Nearly 8% of other introductions are accredited to accidental introductions, such as the escape from aquaculture installations, dispersal through ballast water, or introduced as a contaminant of a consignment of aquaculture species (Gozlan et al., 2010).

The exotic weatherfishes presence in Bita Lake occurred due to an unusual pathway of biological introduction - the practice as “prayer animal release”. Bita Lake is a protected area in the Bitahai Nature Reserve, where intentional introduction of exotic species is strictly forbidden. However, the majority of citizens in

Table 1 Diet composition of *P. chungtienensis* and weatherfishes in Bita Lake

Prey items	<i>P. chungtienensis</i>				Weatherfishes			
	N%	W%	F.O%	IRI	N%	W%	F.O%	IRI
Amphipoda	33.9	51.02	50	4246	42.89	54.11	94.74	9 189.78
Diptera	9.04	2.66	16.67	195.04	37.09	10.06	84.21	3970.5
Trichoptera	53.67	11.78	83.3	5 451.99	1.22	1.01	5.26	11.73
Copepoda	—	—	—	—	9.65	0.7	26.32	272.41
Ciclodocerans	—	—	—	—	4.57	0.35	31.58	155.37
Gastropods	—	—	—	—	4.57	12.43	26.32	447.44
Oligochaeta	1.67	1.93	33.3	119.88	—	—	—	—
Odonata	1.67	3.93	16.67	93.35	—	—	—	—
Algae	35	20.64	100	5564	45	21.34	100	6634
Aquatic plants	16	8.04	66.67	1 602.75	—	—	—	—

the area practice Tibetan Buddhism, when releasing animals into the wild are thought to generate good karma in metempsychosis. Buddhist monks have played an important role in promoting conservation efforts around the world (Chong, 2012). However, religious belief can also lead to unsustainable practices without sound ecological knowledge (Gong et al., 2012). The introduction of exotic weatherfishes into Bita Lake may be a negative example. Moreover, the active release of weatherfishes into the lake is sporadic and private, according to a conservation officer, as the believers usually use coat pockets or backpacks to transport the weatherfishes, and release them into the lake or the inflowing streams.

Although the annual fluctuation from Echo recordings shows little or no significant difference in fish amount between 2009 and 2012 in Bita Lake, the ratio of weatherfishes to *P. chungtienensis* by catch has been increasing since the former were first collected in August, 2009. *Ptychobarbus chungtienensis* is an endemic fish with a current distribution confined to Bita Lake, and its genetic diversity revealed in this study is very low when even comparing the h and π values to the pooled population of the flagship species of conservation, Chinese sturgeon *Acipenser sinensis*, one of the four Category I State protected fishes in China (h , 0.666 vs. 0.949; π , 0.00081 vs. 0.011) (Zhang et al., 2003). By contrast, weatherfishes thrive from the cold temperate to the subtropical regions in eastern Asia, and has been shown to expand its range very rapidly after introduction into new habitats throughout the world (Franch et al., 2008; United States Fish and Wildlife Service, 2012). Another two lakes on the Zhongdian plateau, Shudu Lake and Napa Lake, were once populated by *P. chungtienensis*. However, when various non-native fishes including weatherfishes were introduced into these lakes, the *P. chungtienensis* no longer occurs in them now. Now weatherfishes have been introduced into Bita Lake. Given their very successful survival strategy (United States Fish and Wildlife Service, 2012), and the high degree of dietary overlap with *P. chungtienensis* that we have demonstrated above, we are deeply concerned about the future existence of *P. chungtienensis*.

In order to further preserve *P. chungtienensis* from its last habitat, first of all, more effective measures are imperative to prevent other introduced species from Bita Lake, which need wisdom to balance the existing conflict between conservation and religious faith. Furthermore, continuous periodic monitoring and comparative studies are indispensable to find if there is any other critical factors that will put the *P. chungtienensis* in more danger. Last but not the least, some pre-arranged plans are also necessary before it's too late, such as the timely launching of artificial breeding research of *P. chungtienensis*.

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